Modification of the skin feeding site by tick saliva mediates virus transmission

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Abstract. A tick vector of Thogoto (THO) virus was shown to secrete a factor in saliva which potentiates the transmission of THO virus to uninfected ticks feeding on an apparently non-viraemic host. The effect of the saliva activated transmission (SAT) factor on the virus occurred at the site of inoculation in the skin and was apparent even when the virus was introduced 3 days after the SAT factor. The results suggest that tick saliva can play an important role in disease transmission by virtue of host modification at the site of feeding.
Key words. Tick saliva; virus transmission; host skin modification.

Infected and uninfected vectors of arthropod-borne viruses (arboviruses) feed together on the same vertebrate host. Virus transmission from the infected to the uninfected vector occurs if the vertebrate host on which the vector feeds develops a viraemia characterized by significant levels of virus circulating in the blood[2]. The implications of this dogma are that only viraemic hosts play an important role in the epidemiology of the virus and that, in principle, all the potential vertebrate reservoirs of a virus can be identified by screening for viraemic hosts[3]. Previous work from our laboratory has challenged this premise by demonstrating that a host which is apparently refractory to an arbovirus infection can serve as a medium through which virus is passed from an infected to an uninfected vector.

The latter observations were made with Thogoto (THO) virus, a tick-borne virus of medical and veterinary significance that is distantly related to the influenza viruses[4-7]. At least two African ixodid tick species, *Rhipicephalus appendiculatus* and *Amblyomma variegatum*, are competent vectors of THO virus[8]. Larval and nymphal stages can be infected by feeding on viraemic hamsters. Such ticks, after moulting to the next stage, transmit the virus to uninfected hamsters during feeding. In contrast, if infected ticks feed on guinea pigs, the host does not develop a viraemia detectable by plaque assay or mouse inoculation. But when infected ticks (donors) are allowed to co-feed on guinea pigs with uninfected ticks (recipients), the majority of recipient ticks become infected even though they are physically separated from infected ticks. Indeed, 'non-viraemic transmission' of THO virus involving guinea pigs was more efficient than classical 'viraemic transmission' involving hamsters[9].

The phenomenon of non-viraemic transmission was reproduced by s.i. of tick-infested guinea pigs with THO virus mixed with salivary gland extract (SGE) derived from partially fed (uninfected) female *R. appendiculatus* or *A. variegatum* ticks[10]. The number of recipient ticks which became infected was increased 10-fold compared to ticks which fed on guinea pigs inoculated with virus alone. Potentiation of virus transmission was specific for SGE and was not demonstrated with extracts derived from other tick organs. Maximum enhancement of virus transmission was observed when the inoculum included SGE from *R. appendiculatus* or *A. variegatum* ticks which had fed for 6 or 7 days, respectively, suggesting that the activating factor is not a structural component of the salivary glands. In this report we demonstrate that the enhancing factor is secreted in tick saliva [saliva activated transmission (SAT) factor] and that it interacts with THO virus within the area of inoculation in the skin.

Materials and methods

Cells and virus. BHK-21 and Vero cell cultures were propagated in modified Eagle's medium (EMEM) supplemented with 10% newborn bovine serum (NBS). The Sicilian isolate of THO virus[11] was used throughout the study. The virus was passaged seven times in suckling mice, plaque picked three times in Vero cells, and then passaged three times in BHK-21 cells[12].

Ticks. Laboratory colonies of *R. appendiculatus* and *A. variegatum* were established by feeding all three stages of *R. appendiculatus* and the larval and nymphal stages of *A. variegatum* on Dunkin Hartley guinea pigs; adult *A. variegatum* were fed on New Zealand White rabbits[13].

Collection of saliva. Female *A. variegatum* ticks were removed from guinea pigs at 3, 4, 5, 6, 7, 8, 9, 10, 11, or 13 days after attachment. Saliva was collected by injecting female ticks parenterally with 25 µl/100 mg b.wt of dopamine hydrochloride (2 mM stock solution in 1.2% NaCl). Following injection, ticks were secured dorsal side down to a glass slide, and a glass capillary tube affixed to the slide using modelling clay. The capillary was aligned so as to splay the palps and ensheath the chelicerae of the tick. The slide was then placed at the edge of a heat pad to maintain the tick at approximately 25–30 °C. Secreted saliva was allowed to accumulate in the capillary tube for 30 min (by which time most ticks had ceased secreting), salivary glands were dissected out and both saliva and salivary gland extract (SGE) was clarified by low speed centrifugation prior to storage at −70 °C.

Virus assay. Nymphs were homogenized individually in 1 ml of EMEM containing 10% NBS and antibiotics

appropriate to inhibit bacterial growth. Blood samples were obtained on day 5 post tick attachment, by cardiac puncture from anaesthetized guinea pigs. Titration of blood or tick derived material for virus assay was undertaken using Vero cells incubated at 35 °C for 4 days, prior to fixation and staining.

Time course of virus transmission. Guinea pigs (2 per sample) were infested with approximately 70 uninfected *R. appendiculatus* nymphs. Each guinea pig was inoculated subcutaneously with 5000 plaque forming units (PFU) THO virus and either SGE (20 µg protein) or saliva (10 µg protein) taken from ticks which had fed for a defined period of days; control guinea pigs were inoculated with virus alone. Virus transmission was measured by the number of uninfected ticks that became infected. Ticks were assayed for virus by plaque titration 12 days after engorgement, the time of maximum virus titre[12].

Inoculation of guinea pigs with THO and SGE at different times. Guinea pigs infested with uninfected *R. appendiculatus* nymphs were either inoculated with virus on day 0, and with SGE at 24 h, 48 h, 72 h and 96 h, or, conversely, inoculated with SGE on day 0 and at 24 h, 48 h, 72 h and 96 h with THO virus; control guinea pigs were inoculated with either a mixture of THO virus and SGE simultaneously or with virus alone. Virus or SGE was inoculated along the posterior line on the back of the guinea pigs using a 25 g 5/8-inch butterfly needle. The needle was held in place with surgical tape and the subsequent inoculation made through the same needle.

Location of THO virus in guinea pigs. Four guinea pigs infested with uninfected *R. appendiculatus* nymphs were inoculated with either a mixture of THO virus plus SGE or with virus alone. Animals were killed on day 5 post-inoculation, the ticks removed, and selected organs (brain, axillary lymph nodes, heart, lung, liver, spleen, ovary, kidney, skin [excised at site of inoculation], blood) homogenised and assayed for virus by plaque titration or inoculation of 2-day-old mice (PO strain, University of Oxford).

Titration of blood samples in mice. Two-day-old mice were inoculated intracerebrally with 10 µl of diluted blood or selected organ homogenates. When mice displayed clinical signs of infection, they were humanely killed and their brains dissected out. Individual brains were homogenized in 1 ml of phosphate buffered saline (PBS) and centrifuged at 4000 rpm for 10 min to remove large debris. Plaque neutralization assays[12] were undertaken to confirm that the brains of affected mice contained THO virus. Minimum detectable titres of THO virus were 20 PFU/ml blood by plaque titration and 1.0 $\log_{10}$ mouse median lethal dose (LD_{50})/0.01 ml blood/organ homogenate by inoculation of mice.

Results and discussion

Enhancement of THO virus transmission was observed when the inoculum contained saliva or SGE collected from ticks which had fed for a period of 4–7 days; a total of 203/339 (60%) and 170/335 (51%) of ticks, respectively, acquired virus during this period (table 1). Enhancement of virus transmission was also observed when the inoculum included day 8 SGE (61/100, 61% of ticks acquired THO virus) but was less when it included day 8 saliva (11/50, 22% of ticks acquired virus). By day 9, the enhancing effect of saliva and SGE was significantly reduced in all but one of the guinea pigs. The apparent disparity in the day 9 saliva (72% of ticks on one guinea pig became infected) was attributed to individual variation between animals resulting from the fact that the guinea pigs were outbred to maintain genetic diversity in the colony. Enhancement of virus transmission was not observed when samples were obtained from ticks which had fed for 3 or 13 days. The results demonstrate that overall secretory dynamics of SAT factor in tick saliva parallel closely its synthesis in the salivary gland (fig. 1) and strongly suggest that the two are related.

Absence of SAT factor activity in day 8 saliva was recorded in a preliminary study. None of the 90 ticks which fed on two guinea pigs inoculated with a mixture of virus and day 8 saliva acquired virus. To investigate whether this observation was due to a change in salivary gland activity at day 8 of feeding, the rate of salivary fluid secretion was determined for ticks from which saliva was

Table 1. Comparison of SAT factor activity in the saliva and salivary glands derived from *A. variegatum*

Treatment*		Mean weight of ticks (mg) donating saliva ± SEM (n) §	No. infected/ total no. recipient nymphs†	
			Saliva	SGE
Day 3	(a)	25.0 ± 2.0 (27)	8/40	5/37
	(b)		13/50	1/24
Day 4	(a)	31.0 ± 3.0 (53)	26/49	26/50
	(b)		11/20	7/15
Day 5	(a)	35.5 ± 2.0 (30)	22/40	18/40
	(b)		30/50	20/50
Day 6	(a)	49.3 ± 1.1 (26)	38/50	25/40
	(b)		23/40	29/50
Day 7	(a)	68.5 ± 2.9 (31)	23/40	20/30
	(b)		30/50	25/40
Day 8	(a)	84.0 ± 2.0 (17)	7/50	33/50
	(b)		4/50	28/50
Day 9	(a)	143.0 ± 4.0 (15)	11/50	11/50
	(b)		36/50	12/50
Day 10	(a)	154.0 ± 8.0 (16)	8/40	8/50
	(b)		11/50	10/50
Day 11	(a)	373.0 ± 32.0 (8)	13/40	17/50
	(b)		10/30	13/50
Day 13	(a)	251.0 ± 8.0 (7)	9/50	5/50
	(b)		6/40	3/40

* Virus inoculum mixed with saliva or SGE collected from adult female *A. variegatum* that had fed for a defined period on uninfected guinea pigs. Each mixture was administered to two guinea pigs, (a) and (b), infested with uninfected *R. appendiculatus* nymphs; control guinea pigs received virus alone. § Ticks used to prepare the inoculum for guinea pig replicates (a) and (b). † The number of uninfected recipient nymphs that became infected after feeding on guinea pig replicates (a) and (b) inoculated with a mixture of virus and either saliva or SGE. The numbers of infected recipient ticks from control guinea pigs were (a), 2/31, and (b), 0/9. Virus titres of all recipient ticks were similar, ranging from 3.0–4.1 $\log_{10}$ PFU/tick, indicating that the amount of virus taken up by the ticks was not affected by the treatment. Virus was not detected in whole blood collected from the guinea pigs 4 or 5 days after inoculation (< 20 PFU/ml).

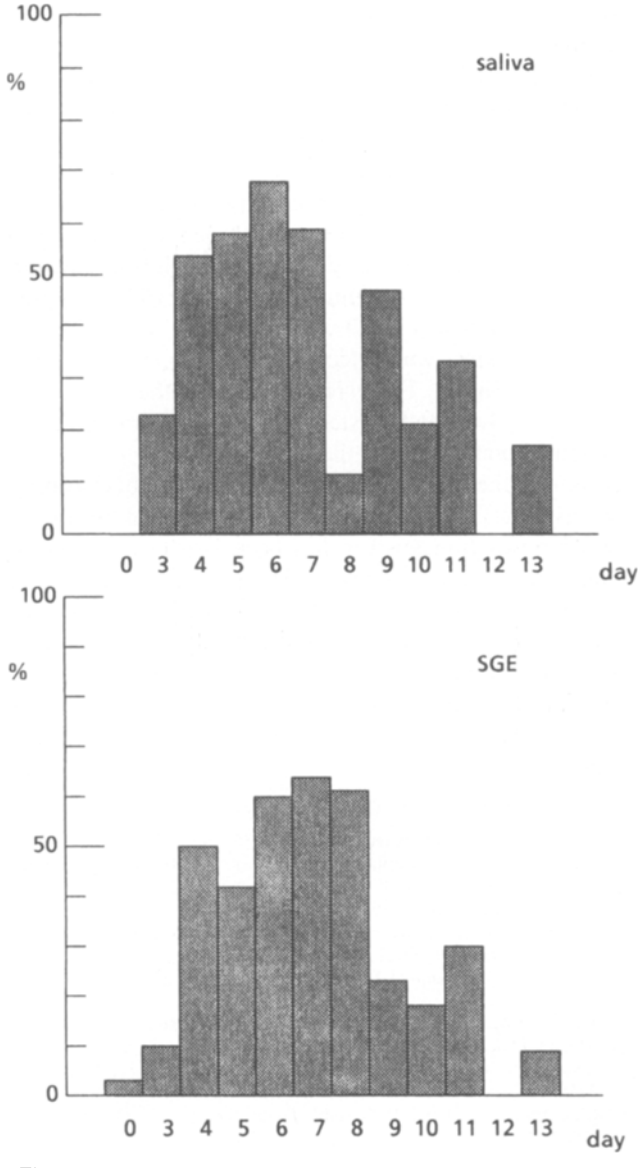

Figure 1. Comparison of the mean percentages of *R. appendiculatus* nymphal ticks that became infected while feeding on guinea pigs inoculated with THO virus mixed with either saliva or the respective salivary glands of uninfected adult female ticks that had been feeding for the indicated number of days. The data are derived from table 1.

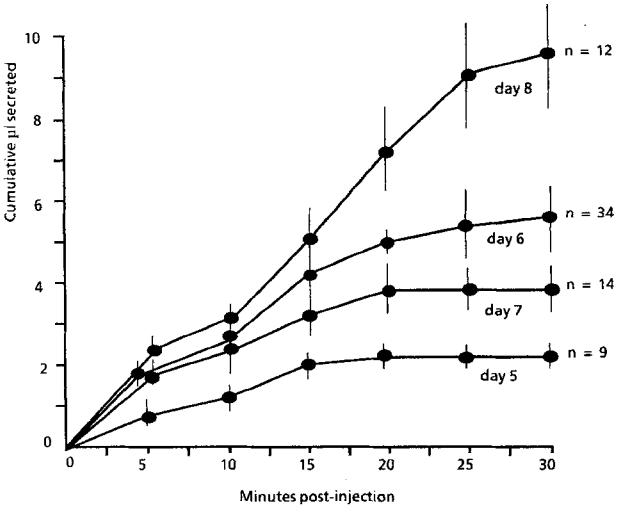

Figure 2. Secretory response over 30 min of *A. variegatum* female ticks contributing saliva for the assay of SAT factor activity. In most cases secretion of saliva ceased by 30–45 min post-injection of dopamine. The overall increase in fluid secretory rates from days 5–8 (excepting day 7) was attributed to the general increase in tick size (see table 1). Mean ± SEM are indicated and the number of replicates (n) at each time point per day of feeding.

collected (as in table 1). Less than 2 µl of saliva were secreted in 30 min by ticks which had fed for 5 days, whereas ticks which had fed for 8 days secreted approximately 9 µl of saliva. These results indicate that the apparent change in SAT factor activity was not due to a reduced capacity of day 8 ticks to secrete saliva when challenged with dopamine.

Several criteria for establishing dopamine as a natural neurotransmitter substance initiating salivary fluid secretion in ticks have been satisfied [14]. The amount of dopamine which crosses the salivary gland epithelium during fluid secretion is not known, and so we cannot tell to what extent the dopamine content of saliva might be responsible for the SAT-factor activity. However, SAT factor activity was not correlated with volume of saliva (and hence amount of dopamine) injected into the guinea pigs. For example, guinea pigs inoculated with day 8 saliva (little SAT factor activity detected) received 180 µl saliva, whereas guinea pigs inoculated with day 7 saliva (maximum SAT factor activity) received only 50 µl of saliva. Thus, it seems unlikely that SAT of the virus was induced by dopamine per se.

Detection of the SAT factor in saliva confirms that it is not a structural component of tick salivary glands but rather that the SAT factor is introduced into the feeding lesion during normal tick feeding. Furthermore, studies have demonstrated that enhancement of virus transmission is a localized rather than a generalized response in the host and is not achieved by direct action on the virus, e.g. by proteolysis [15].

To determine whether the SAT factor has an indirect effect on the virus at the site of inoculation, the time between virus inoculation and SGE injection was varied (table 2). Similar levels of enhancement of virus transmission were observed when the interval between virus inoculation and SGE was 72 h or less (GP 1–6, 51–65% of ticks acquired THO virus); after this time the enhancing effect was reduced (GP7–8, 23% ticks infected). Likewise in the converse experiment, i.e. guinea pigs inoculated with THO virus followed by SGE (GP 9–16), levels of enhancement were similar at inoculation intervals of 24 h and 48 h (50% and 62% ticks infected), but significantly reduced at 72 h and 96 h (16% and 4% infected, respectively).

Although inoculation by needle and syringe does not mimic the skin modification which is caused by feeding ticks, the above observations indicate that the SAT factor induces a comparatively long-term effect in the area of

Table 2. Oral infection of *R. appendiculatus* nymphs feeding on guinea pigs inoculated with THO virus and SGE into the same skin site but at different time intervals.

Guinea pig No.	Treatment*	No. infected/ no. tested	% infected	Virus titre ± SEM†
1–2	D0 SGE, D1 THO	36/70	51	4.2 ± 0.9
3–4	D0 SGE, D2 THO	39/60	65	3.4 ± 0.7
5–6	D0 SGE, D3 THO	34/60	57	3.7 ± 0.6
7–8	D0 SGE, D4 THO	16/70	23	4.6 ± 0.8
9–10	D0 THO, D1 SGE	40/80	50	3.4 ± 0.8
11–12	D0 THO, D2 SGE	74/120	62	4.3 ± 0.4
13–14	D0 THO, D3 SGE	11/70	16	3.6 ± 0.6
15–16	D0 THO, D4 SGE	2/50	4	3.8, 4.4
17–18	D0 THO + SGE	42/90	47	4.5 ± 0.9
19–20	D0 THO	4/70	6	3.8 ± 0.8

*Guinea pigs were inoculated on day 0 with SGE and on either day 1, 2, 3, or 4 with THO virus (GP 1–8), or inoculated with THO virus on day 0 and on either day 1, 2, 3 or 4 with SGE (GP 9–16). Control animals were inoculated with either THO and SGE at the same time (GP 17–18), or with virus alone (GP 19–20). †The geometric mean titre ($\log_{10}$ PFU/tick) ± standard error of the mean (SEM) was calculated from those ticks found to contain virus.

inoculation and that this host modification is beneficial for the virus. Thus, the SAT factor appears to have an indirect effect on the virus. Furthermore, THO virus is able to persist in the host for up to 48 h after inoculation. Studies with THO virus indicate that virus is first delivered by *R. appendiculatus* within 48 h of tick attachment[12], whereas the SAT factor is not secreted until about 4 days post-attachment of ticks. Hence the results obtained when virus is followed by SGE is more indicative of the situation observed experimentally with cofeeding ticks on a non-viraemic host, although ticks deliver virus and SAT-factor over a prolonged period compared with syringe inoculation. However, in nature not all ticks attach to a host at the same time and this, together with the tendency for ticks to cluster together on a host, may facilitate SAT.

The introduction of tick salivary antigens into the feeding lesion is a potent mediator of host reactions[16]. To counteract these reactions, tick saliva possesses many pharmacological substances including anti-hemostatic, vasodilatory, anti-inflammatory and immunosuppressive activity[17]. Thus, a pathogen that is injected into the feeding site of an arthropod vector may benefit from a skin site which is profoundly altered by the effects of vector saliva. This has been demonstrated in the transmission of *Leishmania major* by sandflies. A peptide extracted from the salivary glands of sandflies exacerbated the cutaneous lesion in experimental mice[18]. A similar but less pronounced effect was observed using calcitonin gene-related peptide, CGRP[19]. Physico-chemical properties of the SAT factor of *R. appendiculatus* indicate that it is a protein[20]. However, replacement of the SAT factor by CGRP did not enhance THO virus transmission (Jones, unpublished observation).

Although the studies reported here demonstrate that THO virus transmission is mediated by tick saliva, the question of how the virus passes from infected to uninfected ticks cofeeding on a guinea pig (even when the cohorts are 160 mm apart[15]), remains unanswered. To address this question, the location of THO virus in guinea pigs was examined. Of the ticks which fed on 2 guinea pigs inoculated with virus plus SGE, 65/109 (60%) acquired virus compared to 10/90 (11%) of ticks which fed on 2 guinea pigs inoculated with virus alone. Virus was not isolated from any of the organ homogenates by plaque titration, but when inoculated into 2-day-old mice, virus was detected in the drainage lymph nodes and spleen from animals inoculated with virus plus SGE (confirmed as THO virus by plaque neutralization assay). No virus was detected in guinea pigs inoculated with virus alone. These results suggest that SAT may involve the lymphatic system and perhaps viral infection of some components of the immune system.

Although the skin is the organ via which blood-feeding vectors infect their vertebrate hosts, the role of this organ in arbovirus transmission has been largely overlooked. The skin contains a complex of cells, the skin associated lymphoid tissue (SALT), which enables it to react autonomously to immune challenge[21]. The involvement of SALT, including specific trafficking of lymphocytes, may explain why non-viraemic (i.e. saliva activated) transmission of THO virus is so efficient in the guinea pig-tick model.

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1 Please direct reprint requests to Patricia A. Nuttall.

2 Karabatsos, N., in: International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates. American Society of Tropical Medicine and Hygiene, 1985.
3 World Health Organization, Arthropod-borne and rodent-borne viral diseases. WHO Technical Report, No. 719, 1985.
4 Haig, D. A., Woodall, J. P., and Danskin, D., J. gen. Microbiol. *38* (1965) 389.
5 Davies, F. G., Soi, R. K., and Wariru, B. N., Vet. Rec. *115* (1984) 654.
6 Moore, D. L., Causey, O. R., Carey, D. E., Reddy, S., Cooke, A. R., Akinkugbe, F. M., David-West, T. S., and Kemp, G. E., Ann. trop. Med. Parasit. *69* (1975) 49.
7 Staunton, D., Nuttall, P. A., and Bishop, D. H. L., J. gen. Virol. *70* (1989) 2811.
8 Jones, L. D., Davies, C. R., Steele, G. M., and Nuttall, P. A., Med. Vet. Ent. *3* (1989) 195.
9 Jones, L. D., Davies, C. R., Steele, G. M., and Nuttall, P. A., Science *237* (1987) 775.
10 Jones, L. D., Hodgson, E., and Nuttall, P. A., J. gen. Virol. *70* (1989) 1895.
11 Albanese, M., Bruno-Smiraglia, C., Di Cuonzo, G., Lavignino, A., and Srihongse, S., Acta virol. *16* (1972) 267.
12 Davies, C. R., Jones, L. D., and Nuttall, P. A., Am. J. trop. Med. Hyg. *35* (1986) 1256.
13 Jones, L. D., Davies, C. R., Steele, G. M., and Nuttall, P. A., Anim. Tech. *39* (1988) 99.
14 Kaufman, W. R., and Wong, D. L. P., Eur. J. Pharmac. *87* (1983) 43.
15 Jones, L. D., and Nuttall, P. A., Trans. Roy. Soc. Trop. Med. Hyg. *83* (1989) 712.
16 Wikel, W. K., in: Current Topics in Vector Research, p. 249. Ed. K. F. Harris. Praeger Publishers, New York 1983.
17 Neitz, A. W. H., and Vermeulen, N. M. J., Onderstepoort J. vet. Res. *54* (1987) 443.
18 Titus, R. G., and Ribeiro, J. M. C., Science *239* (1988) 1306.
19 Ribeiro, J. M. C., Vachereau, A., Modi, G. B., and Tesh, R. B., Science *243* (1989) 212.
20 Jones, L. D., Hodgson, E., and Nuttall, P. A., Arch. Virol., Suppl. 1 (1990) 227.
21 Streilein, J. W., J. Invest. Dermatol. *85* (1985) 10s.

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